Circumferential heterogeneity in the abdominal aortic aneurysm wall composition suggests lateral sides to be more rupture prone

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Objective: **The purpose of this study was to identify local differences in inflammation and tissue degradation within the circumference of the abdominal aortic aneurysm (AAA).**

Background: **AAAs have the potential to rupture, and it is unknown why this predominantly occurs at the posterolateral wall. Blood flow dynamics likely influence rupture location but do not explain the whole picture, suggesting that other factors inside the AAA wall have a prominent role.**

Methods: **As part of the Aneurysm-Express study, full thickness circular biopsy specimens of AAAs from 25 patients were obtained during surgery according to a standardized protocol. Tissue from the dorsal, ventral, and lateral sides was processed for histology and protein extraction. Levels of matrix metalloproteinase (MMP)-2 and MMP-9 and various cytokines were measured.**

Results: **Lateral AAA sites, when compared with the ventral and dorsal segments, showed more microvessels (median [interquartile range] per mm^2, 91.8 [72.6-124.6] vs 73.9 [63.0-108.0] and 73.6 [52.7-109.5]; $P = .013$ and $P = .005$, respectively) and more adventitial inflammation (16.1% [13.5%-24.7%] vs 5.8% [2.8%-18.6%] and 6.3% [4.3%-13.5%]; $P = .001$ and $P < .001$, respectively). We observed a higher active MMP-9 (0.139 [0.059-0.339] ng/mL vs 0.060 [0.000-0.157] ng/mL and 0.045 [0.000-0.147] ng/mL; $P = .001$ and $P = .014$, respectively) and higher interleukin-8 (28.644 [11.921-62.587] pg/mL vs 16.442 [4.300-34.130] pg/mL and 18.258 [8.273-44.989] pg/mL; $P < .001$ and $P = .010$, respectively).**

Conclusion: **Biopsy specimens of the ventral AAA wall do not optimally reflect the magnitude of inflammatory processes in the AAA. The lateral sides of the AAA contain more microvessels, more inflammatory cells, more active proteases, and higher cytokine levels. These results suggest that the lateral aortic regions are more rupture-prone and may better reflect the inflammatory status in histopathologic examinations. (J Vasc Surg 2012;55:203-9.)**

Clinical Relevance: **AAAs rupture predominantly at the posterolateral wall; however, current tissue research focuses on the ventral wall. This study indicates increased vulnerability of the posterolateral wall, which might better represent the inflammatory status of the wall instead of the current standard. This can be relevant for trials involving pharmaceutical stabilization of the aneurysm by diminishing wall inflammation. In addition, this study feeds the hypothesis that AAA growth might be more pronounced laterally, which is important for the orientation of diameter measurements.**

Abdominal aortic aneurysm (AAA) rupture has a 30-day mortality rate of more than 60%.[1] Owing to aging of the population in Western countries, AAA disease is becoming more prevalent, with a subsequent rise in associated mortality.[2-4] The pathogenesis of AAA formation still remains to be clarified, which may be partly explained by the limited number of available animal models for AAA formation. In addition, pathologic studies on human aneurysm tissue are restricted to small sample numbers. The availability of AAA tissue samples may become more limited in the near future due to the increasing application of endovascular repair of AAAs.[5]

An AAA is a large structure, which by definition exceeds 3 cm but can range up to 17 cm in diameter.[6] Histopathologic research on human AAAs has mainly been focused on a paraincisional part of the ventral AAA wall. Despite this focus, it is unknown whether a ventral wall specimen is representative for the entire circumference of the AAA.[7] In addition, aneurysm rupture is a localized process, predominantly occurring at the posterolateral part of the AAA, as reported by autopsy studies.[6,8]

Our group previously showed that the aortic pulsatile distention is asymmetric, indicating differing function and

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thereby wall composition for the different regions within the circumference.[9] The morphology of AAAs is asymmetric. Blood flow patterns and wall stress measurements show pronounced local differences, with subsequent computational models indicating that geometry contributes more to AAA expansion than patient risk profile.[10] On positron emission tomography-computed tomography (PET-CT), metabolic activity inside the AAA wall also shows a heterogeneous pattern,[11] all of which is supportive for presence of regional variance in wall composition within the AAA.

Our aim was to investigate local circumferential differences in aneurysm wall components at the level of the maximal diameter. We had a specific interest in the extent of inflammation and protease levels because this potentially causes wall vulnerability and might accelerate AAA expansion and increase rupture risk. We hypothesized that lateral segments would differ from ventral and dorsal segments given the asymmetric distention, morphology, wall stress, and rupture location.

METHODS

Aneurysm-express study. The present work is part of the Aneurysm-Express Biobank study, a prospective cohort study collecting aneurysm tissue from patients undergoing open AAA repair. Its design was described previously.[12] Briefly, indication for open surgery is set according to international standards.[13] The ethical review boards of the two participating centers approved this study. Consecutive patients scheduled for open AAA surgery were enrolled, and written informed consent was obtained from all patients. Cardiovascular risk factors, medical history, and medication use were recorded at baseline. Aneurysm diameter was determined by CT angiogram. Patients completed an extensive questionnaire, based on the Rose Cardiovascular Survey.[14]

Tissue collection and processing. During elective open AAA repair, a full thickness circular specimen was obtained at the site of the maximum diameter, shortly after placing the proximal and distal clamps and sewing in the prosthesis, and when local adhesions were absent. This tissue was immediately processed in the operating room according to a strict protocol, as shown in Fig 1. We maintained an equal distance between the different specimens that were designated for histology and protein extraction. Specimens obtained from the ventral, dorsal, and lateral sides were processed separately.

For a previous study, we collected postmortem normal infrarenal aortic specimens during autopsy.[12] Aortas exhibiting aneurysms or rupture were excluded. The entire circumference was used for histology.

The segments designated to histologic assessment were fixed in 4% formaldehyde and embedded in paraffin. Consecutive slides were stained with hematoxylin and eosin (H&E), elastin von Gieson (EvG), picrosirius red, and antibodies against α-actin, von Willebrand factor (vWF), CD68, CD45, CD3, CD20, and CD138. Extracellular matrix components were semiquantitatively scored as (1) minor or (2) moderate to heavy staining in intima, media, and adventitia separately for collagen (picrosirius red) and smooth muscle cells (SMCs; α-actin). Elastin degradation was scored as the estimated percentage of disruption of elastin fibers.

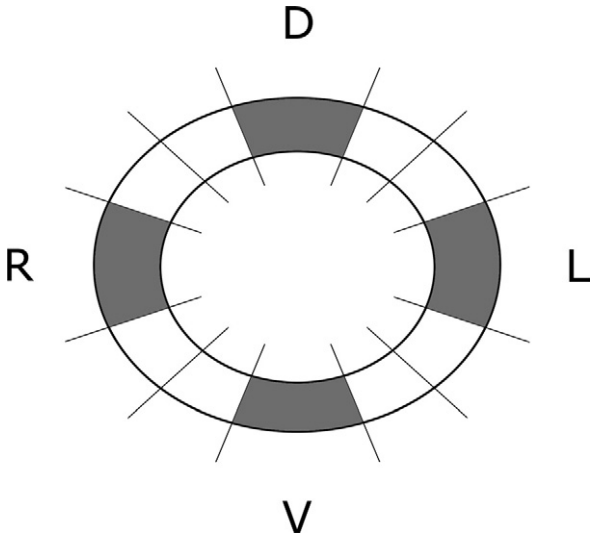

Fig 1. Schematic overview shows abdominal aortic aneurysm (AAA) tissue collection and processing. A circular biopsy specimen at the site of maximum diameter was collected and cut into segments as illustrated. Dorsal (*D*), ventral (*V*), left (*L*), and right (*R*) lateral sides were identified. For each side, a segment of full-thickness AAA wall was processed (*gray area*) for histology and for protein extraction. Other segments were stored for future use.

Different components of the inflammatory infiltrate were scored as (1) minor or (2) moderate to heavy staining in intima and media combined and in the adventitia separately. Minor staining was defined as fewer than 100 positively stained cells per representative high power field at ×100 magnification, and moderate to heavy staining was defined as more than 100 positively stained cells meeting the same conditions. This was performed for macrophages (CD68), total lymphocytes (CD45), T lymphocytes (CD3), B lymphocytes (CD20), and plasma cells (CD138).

Histologic examination was performed by two independent observers (RH, AV), who were blinded from clinical data and laboratory results. In case of discrepancies in judgment, sections were reanalyzed with consensus being reached in all cases.

The adventitial infiltrate was also quantitatively measured. For this analysis, we scanned all H&E and EvG slides using a ScanScope XT scanner (Aperio, Vista, Calif). This method of digitalizing slides in high resolution in our institution was published previously.[15] EvG staining was used to identify the media that formed the inner border of the adventitia, whereas perivascular fatty tissue defined the outer border of the adventitia. Aperio ImageScope software was used to define and measure surface areas of the inflammatory infiltrates and of the total adventitia at ×100 magnification on the H&E slides. The percentage of the adventitia covered with inflammation was quantified by dividing

the area covered with inflammatory infiltrates by the total adventitial surface area.

Staining with vWF was used to measure vessel density, as described previously.[16] Briefly, five hotspots were identified in the media and adventitia, and vWF-positive microvessels were counted at ×100 magnification. Subsequently, the number of microvessels per square millimeter was calculated.

Adjacent specimens appointed for protein extraction were snap-frozen with liquid nitrogen and stored at −80°C. These segments were later crushed in liquid nitrogen, and protein was isolated as described before.[12]

Matrix metalloproteinase (MMP) activities were determined by using the Amersham MMP-2 and MMP-9 Biotrak Activity Assay System (General Electric Healthcare Ltd, Amersham, United Kingdom). Osteopontin (OPN) levels were measured by using enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, Minn). Levels of interleukin (IL) 1β, 2, 4, 5, 6, 8, 10, 12p70, interferon γ, and tumor necrosis factor (TNF)-α and β were quantified in the aneurysmal wall by fluorescent bead immunoassay (Bendermed Systems, Vienna, Austria). All described procedures complied with the manufacturers' protocols.

Statistical analysis. Discrete variables are shown as frequencies and percentages of the total. Continuous variables are shown as median and interquartile range. For multiple, paired continuous data, a Friedman test was used to determine differences among groups. If the result was significant, the Wilcoxon sign test was used to determine which pairs differed. Likewise for discrete paired data, first a Cochrane Q test was performed and, if significant, was followed by a McNemar χ^2 test. Values of $P < .05$ were considered significant. All analyses were performed with SPSS 17 software (SPSS, Chicago, Ill).

RESULTS

None of the patients had previous retroperitoneal surgery. Two patients were excluded due to the presence of adhesions. Circular AAA specimens were collected from 25 patients whose baseline characteristics are reported in Table I. Symptoms attributable to the AAA were present in 3 patients; however, these patients neither had a thickened AAA wall on CT nor retroperitoneal fibrosis. The transverse diameter exceeded the anteroposterior diameter by 8% (5%-10%). The lateral segments of the AAAs were pooled for histology and protein analyses.

For a previous study, the entire circumference of normal infrarenal aortas was collected in autopsies of 27 patients (median age, 54 years old; range, 40-69 years old; 14 men). The different regions within the aorta remained unmarked and systematic localized analyses were, hence, impossible. However, a histologic assessment of the 27 specimens did not show any differences in structural and inflammatory characteristics around the circumference. For illustrative purposes, Fig 2 shows a representative photomicrograph of the circumferential homogeneous normal aortic wall.

Table I. Baseline demographics

Patient characteristics[a]	*AAA (n = 25)*
Age, years	66 (64-77)
Male sex	19 (76)
Current smoker	10 (40)
Diabetes type 2	5 (20)
Hypertension	18 (72)
Coronary artery disease	4 (16)
Peripheral artery disease	6 (24)
Chronic obstructive pulmonary disease	2 (8)
Body mass index, kg/m^2	24.7 (22.8-27.1)
Serum creatinine, μmol/L	90 (81-117)
Aneurysm diameter, mm	60 (54-70)
Symptoms attributable to the AAA	3 (12)
History of any other aneurysm detected	2 (8)
Statin use	20 (80)
Aspirin use	16 (64)
ACEI use	8 (32)
Angiotensin II receptor blocker use	4 (16)
Hospital stay, days	10.0 (8.3-13.5)

AAA, Abdominal aortic aneurysm; *ACEI,* angiotensin-converting enzyme inhibitor.
[a]Data are presented as number (%) or as median (interquartile range).

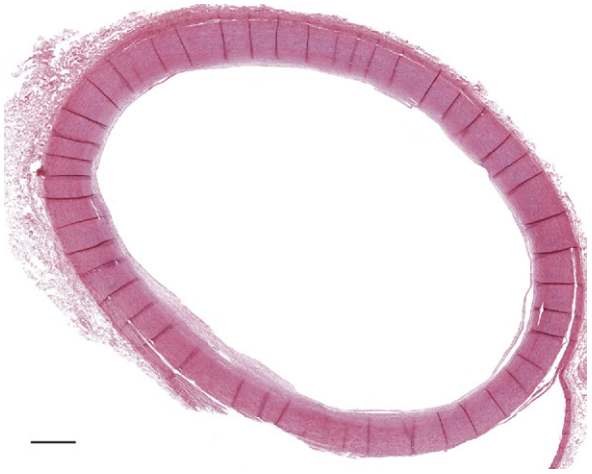

Fig 2. Overview of the circumference of a normal aorta in hematoxylin and eosin (H&E) staining. Representative photomicrograph shows the homogeneous distribution of structural components and lack of inflammation in the normal infrarenal aorta. The *scale bar* represents 1 mm.

Detailed histologic characteristics for the different sites and results of the paired analyses of the groups are summarized in Table II. Lateral and dorsal AAA segments more frequently revealed a cholesterol-rich lipid core compared with the ventral wall (56% and 48% vs 24%; $P = .016$ and $P = .031$). Elastin content and SMCs in the media were similarly degraded in all groups. Collagen quantity was lower in the adventitia of the lateral wall ($P = .065$) and the dorsal wall ($P = .008$) than in the ventral wall. The lateral part of the AAA revealed higher amounts of microvessels per square millimeter compared with the ventral (24% higher; $P = .013$) and dorsal (25% higher; $P = .005$) parts.

Table II. Histologic characteristics

AAA wall characteristics[a]	*Ventral*	*Lateral*	*Dorsal*	P *value*
Structural components				
Intima				
Cholesterol core, presence	6 (24)	14 (56)	12 (48)	.032[b,c]
Collagen	9 (36)	3 (12)	6 (24)	.235
SMCs	6 (24)	3 (12)	3 (12)	.904
Media				
Elastin disruption, %	55.0 (2.5-80.0)	59.5 (41.3-95.0)	60.0 (30.0-96.8)	.475
SMCs	7 (28)	2 (8)	5 (20)	.307
Adventitia				
Collagen	20 (80)	13 (52)	10 (40)	.023[d]
SMCs	10 (40)	8 (32)	7 (28)	.423
Microvessels per mm^2	73.9 (63.0-108)	91.8 (72.6-124.6)	73.6 (52.7-109.5)	.035[b,c]
Inflammation				
Intima/media				
Lymphocytes	0 (0)	0 (0)	0 (0)	> .99
T lymphocytes	0 (0)	0 (0)	0 (0)	> .99
B lymphocytes	0 (0)	0 (0)	0 (0)	> .99
Plasma cells	0 (0)	0 (0)	0 (0)	> .99
Macrophages	7 (28)	8 (32)	8 (32)	.846
Adventitia				
Covered by inflammation, %	5.8 (2.8-18.6)	16.1 (13.5-24.7)	6.3 (4.5-13.5)	< .001[b,c]
Lymphocytes	10 (40)	18 (72)	9 (36)	.001[b,c]
T lymphocytes	9 (36)	14 (56)	6 (24)	.013[c]
B lymphocytes	12 (48)	18 (72)	8 (32)	< .001[b,c]
Plasma cells	4 (16)	4 (16)	5 (20)	.846
Macrophages	1 (4)	4 (16)	5 (20)	.309

AAA, Abdominal aortic aneurysm; *SMC,* smooth muscle cell.

[a]Categoric data are presented as number (%) of heavy staining as opposed to minor staining, unless otherwise indicated. Continuous data are presented as median (interquartile range).

P value results from a paired analysis of the groups, when $P < .05$ a post hoc test was used to determine which groups differed: [b]lateral > ventral; [c]dorsal > ventral; [d]ventral > dorsal; [e]lateral > dorsal.

Analysis of infiltration of inflammatory cells found no differences between sites for the intima and media. In the adventitia, total lymphocytes were higher in lateral than in ventral ($P = .008$) or dorsal ($P = .032$) sites. An analyses of lymphocyte subgroups showed that the most frequently present cell mainly determined the differences: B lymphocytes were more often observed in lateral compared with ventral ($P = .031$) and dorsal wall ($P = .001$; Fig 3). For T lymphocytes, dorsal site staining was lower than in the lateral site ($P = .008$). These results were also reproduced by the quantitative assessment of the percentage adventitia that was covered by inflammatory cells. In lateral segments, the inflammatory infiltrate covered a 2.8-fold larger area of the adventitia than in ventral segments ($P = .001$) and a 2.6-fold larger area than in dorsal segments ($P < .001$; Fig 4).

The histologic differences were accompanied by the measured protease and cytokine concentrations (Table III). Active MMP-2 levels were higher in lateral segments compared with dorsal ($P = .032$), and active MMP-9 was 2.3 times higher in lateral sites than in ventral sites ($P = .001$) and 3.1 times higher than in dorsal sites ($P = .014$). OPN concentrations were also elevated in lateral segments compared with ventral ($P = .004$) and had a higher tendency compared with dorsal ($P = .058$). The cytokine with the highest measured levels, IL-8, was in the lateral sites, 74% higher than in the ventral sites, and 57% higher than in the dorsal sites ($P < .001$ and $P = .010$, respectively).

DISCUSSION

Current research on human AAA tissue is based on specimens collected from part of the ventral wall during open surgical repair. The AAA is a large structure, and it is not known whether its constituents are homogeneously distributed. In addition, it is unknown whether the ventral wall, the most regularly studied part of the circumference, indeed represents the magnitude of inflammatory processes within the AAA. To our knowledge, we are the first to report heterogeneity inside the intact AAA: lateral sides exhibit more active inflammation with more microvessels, more inflammatory cells on histology, and higher protease and cytokine levels than the ventral and dorsal walls.

A previously collected sample of normal infrarenal aortas, in which the different regions had not been marked, was used to analyze the heterogeneity around the circumference of the vessel wall. We did not find any variation in the structural or inflammatory components. In regard to previously reported proinflammatory age-related alterations of the aorta,[17] our results suggest that changes are not necessarily restricted to specific segments. This observation differs substantially from findings in patients with developed AAAs, who exhibited more inflammation-related changes in the lateral parts of the aortic wall.

The observed increased quantity of lateral inflammation corresponds well to the most common sites of AAA

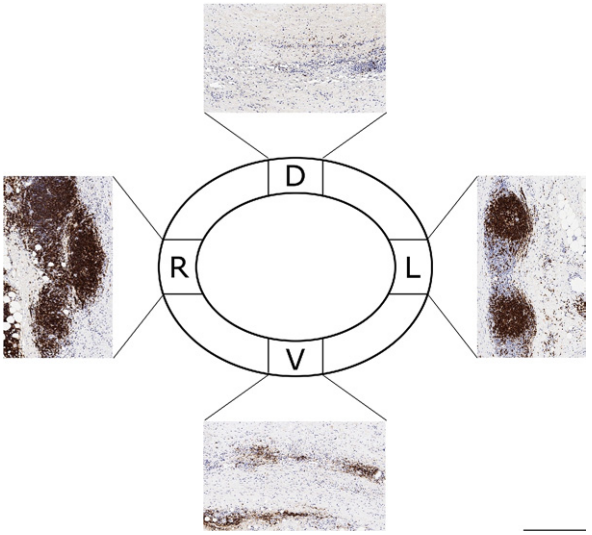

Fig 3. Histologic overview shows the different sites of the abdominal aortic aneurysm in the same patient, stained for CD20-positive B lymphocytes. Representative photomicrographs show different sites within one abdominal aortic aneurysm: dorsal (*D*), ventral (*V*), left (*L*), and right (*R*) lateral segments surround the lumen. The intima is for all four regions located at the luminal side, and the adventitia forms the outer borders of the picture. Note the higher quantity of inflammation in both lateral sides. The *scale bar* represents 1 mm.

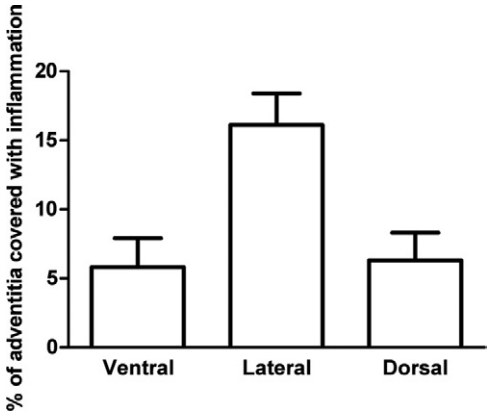

Fig 4. Distribution of the proportion of the adventitia covered with inflammation in relation to different abdominal aortic aneurysm (AAA) sites. This graph shows the distribution of the percentage of adventitia that is covered with inflammation. Note that there is a difference among groups (Friedman test $P < .001$), with lateral inflammation being higher than both ventral (Wilcoxon sign test $P = .001$) and dorsal (Wilcoxon sign test $P < .001$) segments.

rupture, which is posterolateral in 80%.[18] Previous work demonstrated that the sites of rupture revealed higher quantities of MMP-8 and MMP-9 and more neovascularization compared with the intact ventral walls of the same patients.[19,20] This indicates that the laterally increased quantity of microvessels and active proteases in intact AAAs described in present study predisposes this region of the AAA as being more vulnerable to rupture. In addition, we found the number of inflammatory cells and the concentrations of inflammation-stimulating cytokines OPN and IL-8 were all increased at the lateral sites, possibly increasing vulnerability. In previous studies, IL-8 was found to be higher in AAAs when compared to normal aortas, and it was associated with an upregulation of inflammation as well as increased neoangiogenesis.[21,22] OPN has been described to promote inflammation, proteolysis, and atherosclerosis in experimental studies, which are all integrated processes of AAA development and progression.[23] In addition, an animal model linked OPN directly to AAA development; in human OPN in serum was associated with AAA growth.[23,24] A recent study that examined inflammation at the site of rupture reported no difference in IL-6, TNF-α, and IL-1β with the intact ventral wall.[25] Unfortunately, IL-8 and OPN remained unmeasured in that study, which are the most prominent differing cytokines in the present study.

The described vulnerability of the lateral parts of the AAA in this study supports the concept that AAA expansion might be more pronounced laterally, which corresponds with reports in the literature of asymmetric morphology and differences in flow patterns and wall stress.[10] A recent review on computational models for blood flow emphasized the need for taking AAA wall strength into account when analyzing the ability of wall stress to predict vulnerability and rupture.[26] AAA wall strength varies not only between different individuals (based on, for instance, occurrence of risk factors) but also within the same AAA. Furthermore, wall thickness decrease leads to an increase of peak wall stress, and asymmetry of the AAA is strongly related to wall stress distribution.[26] Just as in the current study, the anteroposterior diameter in most AAAs was smaller than the transverse diameter,[27] which alters the distribution of wall stress. Combined with our finding of decreased adventitial collagen dorsally, wall stress further increases in the posterolateral part of the AAA. When combined with the increased vulnerability of the lateral regions of the AAA, this may further accelerate lateral expansion.

Serial studies evaluating local expansion within the circumference are lacking, and it would therefore be interesting to monitor patients with small-diameter AAA over time to assess circumferential growth patterns and, eventually, the site of rupture. This finding may also be relevant for the orientation of measuring maximum AAA diameter because large trials used maximum anteroposterior diameter,[28] transverse diameter, or both.[29] It was furthermore described that diameter asymmetry is relevant for determining rupture risk,[27] and current guidelines do not specify a gold standard for measuring AAA diameter.[13,30]

A specimen of the ventral AAA wall does not necessarily represent the entire AAA wall. Data from small studies using human specimens need to be interpreted carefully, because we have already shown in a limited number of patients that marked regional differences exist. Large hu-

Table III. Protease and cytokine levels

AAA wall characteristics[a]	*Ventral*	*Lateral*	*Dorsal*	P *value*
MMP-2 active	0.112 (0.041-0.180)	0.133 (0.093-0.200)	0.114 (0.051-0.205)	.037[b]
MMP-9 active	0.060 (0.000-0.157)	0.139 (0.059-0.339)	0.045 (0.000-0.147)	.007[b,c]
Osteopontin	0.635 (0.000-1.083)	0.979 (0.242-4.748)	0.470 (0.000-1.101)	.007[c]
IL-1β	0.022 (0.000-0.936)	0.281 (0.000-0.405)	0.000 (0.000-0.317)	.142
IL-2	2.175 (0.959-5.515)	2.697 (0.682-4.049)	1.401 (0.837-4.188)	.258
IL-4	0.423 (0.000-2.152)	0.767 (0.127-1.454)	0.234 (0.033-1.138)	.192
IL-5	1.500 (0.268-4.449)	1.338 (0.609-3.456)	0.797 (0.253-2.438)	.332
IL-6	0.701 (0.129-1.633)	2.320 (0.680-9.911)	1.227 (0.398-3.525)	.782
IL-8	16.442 (4.300-34.130)	28.644 (11.921-62.587)	18.258 (8.273-44.989)	< .001[b,c]
IL-10	0.458 (0.000-1.377)	0.466 (0.221-0.800)	0.285 (0.000-0.785)	.707
IL-12	0.497 (0.000-2.366)	0.650 (0.113-1.505)	0.210 (0.000-1.103)	.292
TNF-α	0.267 (0.000-1.966)	0.238 (0.079-0.823)	0.128 (0.000-0.636)	.600
TNF-β	1.423 (0.000-4.049)	0.780 (0.106-2.162)	0.151 (0.000-2.711)	.560
IFN-γ	0.506 (0.000-2.561)	1.008 (0.471-1.669)	0.533 (0.157-1.184)	.350

AAA, Abdominal aortic aneurysm; *IFN,* interferon; *IL,* interleukin; *MMP,* matrix metalloproteinase; *TNF,* tumor necrosis factor.
[a]Data are presented as median and (interquartile range). Units are ng/mL for MMP-2 and MMP-9 and pg/mL for the other determinants.
P value results from a paired analysis of the groups, when $P < .05$ a post hoc test was used to determine which groups differed: [b]lateral > dorsal; [c]lateral > ventral.

man AAA biobanks are necessary to even the effects of heterogeneity and to provide a reliable source of information for investigating pathogenesis.

Because preventive repair of AAA is associated with considerable cardiovascular morbidity and mortality, possible effects of medication on the AAA wall are receiving much attention. Postponing surgery has a substantial effect on diminishing the occurrence of repair-associated cardiovascular events. For statins, described effects are conflicting based on growth rate follow-up studies and ventral AAA tissue assessment in cohort studies.[31,32] An explorative prospective randomized trial with administration of doxycycline showed a decrease in inflammation and proteases in the ventral wall.[33] The current study, however, raises the question whether the ventral wall alone should be the focus of such trials. The effects of drugs on the lateral sides of the AAA may be more relevant in attenuation of inflammation and protease activity with subsequent growth retardation. This indicates the need for tissue collection at different sites or a more prominent role for imaging of inflammation or proteases as a primary outcome measure.

As with other studies on human AAAs, this study is limited for only being able to assess late-stage disease at one point. However, we aimed to investigate the variance among the different parts within the same AAA and collected these segments at the same time, which makes it unlikely to have influenced present results. The number of patients included in this study did neither allow for reliable analyses of differences in patterns of wall components nor of patient characteristics, such as gender and smoking status.

In conclusion, the constituents of the AAA have a heterogeneous distribution. Commonly used ventral wall specimens do not necessarily resemble the entire AAA wall. Lateral sides of the AAA have more microvessels, more inflammatory cells, higher cytokine levels, and higher levels of active proteases. This infers that these more active regions of the AAA might have a relatively higher contribution to AAA expansion and that these sites are more vulnerable to rupture.

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AUTHOR CONTRIBUTIONS

Conception and design: RH, FM
Analysis and interpretation: RH, GP, AV, MB, HP, JV, FM
Data collection: RH, GP, HP, JV, FM
Writing the article: RH
Critical revision of the article: GP, AV, IH, MB, HP, JV, FM
Final approval of the article: RH, GP, AV, IH, MB, HP, JV, FM
Statistical analysis: RH, GP, IH, MB, FM
Obtained funding: FM
Overall responsibility: RH

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